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Gunilla Larsson

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Fee

PATENT- OCH REGISTRERINGSVERKET
SWEDEN

Postadress/Adress
Box 5055
S-102 42 STOCKHOLM

Telefon/Phone
+46 8 782 25 00
Vx 08-782 25 00

Telex
17978
PATOREG S

Telefax
+46 8 666 02 86
08-666 02 86

TRANSGENIC ANIMAL MODEL

FIELD OF INVENTION

The invention relates to transgenic animal models of Alzheimer's disease and related neurological disorders, which are useful for evaluating potential therapeutic agents and diagnostic markers.

BACKGROUND OF THE INVENTION

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disorder causing cognitive, memory and behavioral impairments. It is the most common cause of dementia in the elderly population affecting roughly 5% of the population above 65 years and 20% above 80 years of age. AD is characterized by an insidious onset and progressive deterioration in multiple cognitive functions. The neuropathology involves both extracellular and intracellular argyrophillic proteineous deposits. The extracellular deposits, referred to as neuritic plaques, mainly consist in amyloid-beta (Aβ) peptides surrounded by dystrophic neurites (swollen, distorted neuronal processes). The Aβ peptides within these extracellular deposits are fibrillar in their character with a β-pleated sheet structure. Aβ in these deposits can be stained with certain dyes e.g. Congo Red and display a fibrillar ultrastructure. These characteristics, adopted by Aβ peptides in its fibrillar structure of neuritic plaques, are the definition of the generic term amyloid. The classic intracellular AD pathologic lesion is the neurofibrillary tangle (NFT) which consists in filamentous structures called paired helical filaments (PHFs) composed of twisted strands of hyperphosphorylated microtubule-associated protein tau. Frequent neuritic plaques and neurofibrillary tangle deposits in the brain are diagnostic criteria for AD, as carried out when the patient has died. AD brains also display macroscopic brain atrophy, nerve cell loss, local inflammation (microgliosis and astrocytosis) and often congophilic amyloid angiopathy (CAA) in cerebral vessel walls.

Two forms of Aβ peptides, Aβ40 and Aβ42, are the dominant species of AD neuritic plaques (Masters et. al., 1985), while Aβ40 is the prominent species in cerebrovascular amyloid associated with AD (Glenner and Wong, 1984). Enzymatic activities allow these Aβ to be continuously formed from a larger protein called the amyloid precursor protein (APP) in both healthy and AD afflicted subjects in all cells of the body. Two major APP processing events β- and γ-secretase activities enables Aβ-peptide production through enzymatic cleavage, while a third one called α-secretase activitites prevents Aβ-peptide by cleavage inside the Aβ-peptide sequence (reviewed in Selkoe, 1994; US5604102). The Aβ42 is forty two amino acid long peptide i.e. two amino acids longer at the C-terminus, as compared to Aβ40. The Aβ42 peptide is more hydrophobic, and does more easily aggregate into larger structures of Aβ peptides such as Aβ dimers, Aβ tetramers, Aβ oligomers, Aβ protofibrils or Aβ fibrils. Aβ fibrils are hydrophobic and insoluble, while the other structures are all less hydrophobic and soluble. All these higher molecular structures of Aβ peptides are individually defined based on their biophysical and structural appearance e.g. in electron microscopy, and their biochemical characteristics e.g. by analysis with size-exclusion chromatography/western blot. These Aβ peptides, particularly Aβ42, will gradually assemble into a various higher molecular structures of Aβ during the life span. AD, which is a strongly age-dependent disorder, will occur earlier in life if this assembly process occurs more rapidly in the brain of that individual. This is the core of the "amyloid cascade hypothesis" of AD which claims that APP processing, the Aβ42 levels and their assembly into higher molecular structures are central cause of all AD pathogenesis. All other neuropathology of AD brain and the symptoms of AD such as dementia are somehow caused by Aβ peptides or assembly forms thereof.

The strongest evidence for the "amyloid cascade hypothesis" comes from genetic studies of individuals in families afflicted by early onset of familial AD as a dominant trait. These studies have revealed that rare mutations in the APP gene are sufficient to generate severe forms of AD. The mutations are clustered in and around Val 717 slightly downstream of the Aβ1-42 C-

terminus (Goate et al., 1991, Chartier-Harlan, et al., 1991, Murrell, et al., 1991) and a unique double mutation (670-671) immediately upstream of the Aβ N-terminus in a Swedish family (Mullan, et al., 1992; US5795963). The APP mutations, which frames the Aβ peptide sequence, were later found to either increase both Aβ40 and Aβ42 production (the "Swedish" mutation; Citron, et al., 1992, Cai et al., 1993), or to increase the ratio of Aβ42/ Aβ40 production and also to generate Aβ peptides that are C-terminally extended to incorporate the pathogenic mutation in the Aβ peptide e.g. Aβ50 (the "717"-mutations are at position 46; Suzuki et al., 1994; Roher et al., 2003). Thus the "717" mutations, in addition to wild-type Aβ40 and wild-type Aβ42, also generate London Aβ peptides (V717I) and Indiana Aβ peptides (V7171F, Aβ46 and longer forms of Aβ) which rapidly forms Aβ fibrils. In contrast, the Swedish mutation only generates increased levels of wild-type Aβ40 and Aβ42 peptides. Early onset familial AD is more often caused by mutations in presenilin 1 (on chromosome 14; US5986054; US5840540; US5449604) and in some cases by mutations in presenilin 2 (chromosome 1). Presenilin 1 and presenilin 2 are both polytopic transmembrane proteins that, together with three other proteins nicastrin, aph1 and pen-2, constitute the necessary functional core of the γ-secretase complex that enables Aβ-peptide formation through enzymatic cleavage of APP (Edbauer et al., 2003). All AD pathogenic mutations in presenilin 1 and presenilin 2 proteins significantly increase Aβ1-42 overproduction (Schuener et al., 1996). Apolipoprotein E (ApoE) is, besides age, the most important risk factor for late-onset AD. There are three variants of the ApoE protein in humans, due to single amino acid substitutions in the ApoE protein. The ApoE4 variant confers increased risk of AD, while the ApoE2 variant is protective as compared to the predominant ApoE3 variant (Strittmatter et al., 1993; Corder et al., 1993). These protein changes are not deterministic, but confer enhanced or decreased susceptibility to develop AD in a population. The ability of the ApoE variants to drive amyloid deposition in APP transgenic mice models of AD is greatest for ApoE4, intermediate for ApoE3 and lowest for ApoE2, suggesting that the AD pathogenic mechanism of ApoE is to enhance Aβ-peptide assembly

and/or amyloid deposition (Fagan et al., 2002). Other proteins such as α_1-antichymotrypsin (Nilsson et al., 2001) and ApoJ/clusterin (DeMattos et al., 2002) also enhance Aβ-peptide assembly and/or amyloid deposition in APP transgenic mice, similar to ApoE. Neprilysin (NEP) and insulin-degrading enzyme (IDE) degrade Aβ peptides and are likely implicated in AD. However, none of these proteins has been proven to be involved in AD by human genetics. A key issue in future AD research is to better understand how enhanced levels Aβ or aggregates thereof cause dementia and functional loss in AD patients. It has been a long-standing belief that the insoluble amyloid fibrils, the main component of the neuritic plaque, are the pathogenic species in AD brain. High concentrations of Aβ fibrils have been shown to be cytotoxic in cell culture models of nerve cells in the brain (Pike et al., 1991; Lorenzo and Yankner et al., 1994). However, the hypothesis of the amyloid fibril as the main neurotoxic species is inconsistent with the poor correlation between neuritic plaque density and AD dementia score and also with the modest signs of neurodegeneration in current APP transgenic mice. Soluble neurotoxic Aβ-intermediate species and their appropriate subcellular site of formation and distribution could be the missing link that will better explain the amyloid hypothesis. This idea has gained support from recent discovery of the Arctic (E693) APP mutation, which causes early-onset AD (W00203911; Nilsberth et al., 2001). The mutation is located inside the Aβ peptide sequence. Mutation carriers will thereby generate variants of Aβ peptides e.g. Arctic Aβ40 and Arctic Aβ42. Both Arctic Aβ40 and Arctic Aβ42 will much more easily assemble into higher molecular structures of Aβ peptides that are soluble and not fibrillar in their structure, particularly Aβ protofibrils. Thus the pathogenic mechanism of the Arctic mutation differs from other APP, PS1 and PS2 mutations and suggests that the soluble higher molecular structures of Aβ peptides e.g. Aβ protofibrils is the cause of AD. It has recently been demonstrated that soluble oligomeric Aβ peptides such as Aβ protofibrils impair long-term potentiation (LTP), a measure of synaptic plasticity that is though to reflect memory formation in the hippocampus (Walsh et al., 2001). Furthermore that oligomeric Arctic Aβ

peptides display much more profound inhibitory effect than wt Aβ on LTP in the brain, likely due to their strong propensity to form Aβ protofibrils (Klyubin et al., 2003).

An animal model of Alzheimer's disease with the features of the human disease is much needed to better understand AD pathogenesis and to evaluate the efficacy of new therapeutic agents. The ideal animal model of AD should generate the complete neuropathology of AD and the clinical phenotype e.g. progressive memory and cognitive dysfunctions. Major progress in this direction has been accomplished using transgenic overexpression of APP harboring AD pathogenic mutations. Current APP transgenic models of AD display important features of AD pathogenesis such as age-dependent and region-specific formation of both diffuse and neuritic plaques in the brain. The amyloid pathology is associated with hyperphosphorylated tau, local inflammation (microgliosis and astrocytosis) and to a variable extent with congophilic amyloid angiopathy (CAA). These models have been generated by very high transgene expression of human APP, particularly in nerve cells of the brain. The transgenes always carries an AD pathogenic mutation. Thus a "717"-APP-mutation (V717F; Games et al. 1995; US2002104104; US5720936; US5811633) or the "Swedish" mutation (KM670/671NL; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; WO 09803644; US2002049988; US6245964; US5850003; US5877399; US5777194) have been used. These APP models of AD do display some memory impairments, but it is unclear how these dysfunctions relate to the dementia of AD patients. Important features of AD pathology that are likely implicated in dementia, such as intracellular tangle formation (NFT), neuronal loss and brain atrophy is lacking in the current APP transgenic models. Double transgenic mice containing both mutant APP and mutant presenilin-1 transgenes develop accelerated amyloid plaques formation, but the animals still display modest mental impairment and still fail to display NFTs, nerve cell and brain atrophy (Holcomb et al., 1998; US5898094; US2003131364). Furthermore the current APP transgenic models likely have low levels of soluble intermediates in the Aβ fibrillization process such as Aβ

protofibrils, which might be of great importance for AD pathogenesis. Several AD pathogenic mutations have previously been combined in one single transgene e.g. the "Swedish" mutation (KM670/671NL) and the"717"-APP-mutation (Indiana, V717F) have been used to enhance and increase formation of fibrillar Aβ peptides and neuritic plaque formation (Janus et al., 2001). Similarly the "Swedish" (KM670/671NL), the "Arctic" (E693G) and a "717"-APP-mutation (London, V717I) have been combined and used to generate earlier and increased plaque formation (Teppner et al., 2003), like those of Swedish+Indiana APP transgenic models (Janus et al., 2001), since the London Aβ peptides will strongly drive Aβ fibril formation (Teppner et al., 2003; Roher et al., 2003). The unique characteristics of Arctic Aβ40 and Arctic A42 to form an abundance of stable protofibrils have been demonstrated (Nilsberth et al., 2001; Lashuel et al., 2003). The marked difference in pathology in human AD brain between carriers of the London APP mutation (Lantos et al., 1992; Cairns et al., 1993) and Arctic APP mutation (Bogdanovic et al., 2002) reinforce the distinction in the chemical characteristics of London Aβ peptides and Arctic Aβ peptides for neuropathology.

There is still a great need for animal models suitable for studying Alzheimer's disease, especially for models mimicking the human pathology as closely as possible.

SUMMARY OF THE INVENTION:

In view of the shortcomings of prior art models, the object of the invention is to provide transgenic animal models that better reflect features of AD in human patients. The current invention describes a different AD transgenic model that mainly forms stable Aβ Arctic peptides to generate early soluble oligomeric and protofibrillar Aβ Arctic peptide-driven pathology, including such Aβ aggregation also inside neurons of the brain and reduced brain weight to better reflect the human AD pathology and its atrophic changes.

This pathology occurs in addition to extracellular plaque formation, which is also found in the APP transgenic mouse model described in the invention.

The invention provides a method of preparing a transgenic non-human animal, preferably a mouse, harboring a transgene encoding amyloid precursor protein (APP) comprising both the Arctic mutation (E693G) and any other APP mutation or APP mutations that will enhance Aβx-40 and/or Aβx-42 Arctic peptides and Aβ Arctic protofibril production and will confer an early soluble oligomeric and protofibrillar Aβ Arctic peptide-driven pathology, including Aβ aggregation inside neurons of the brain to better reflect the human AD pathology. The APPs comprise a group of ubiquitously expressed transmembrane glycoproteins whose heterogeneity arises from both alternative splicing and post-translational processing [Selkoe, D. J. (1994), NCBI accession nr P05067, SEQ ID NO: 1]. Apart from the 751-and 770-residue splice forms expressed in non-neuronal cells throughout the body, neurons most abundantly express the 695-residue isoform. All isoforms are the precursors of various metabolites that result from different proteolytic cleavage induced by physiological or pathological conditions. The APP itself, as used according to the principles of this invention, can be any of the alternative splice forms of this molecule and may be used either as a glycosylated or non-glycosylated form.

Further, the invention provides a method of preparing a transgenic non-human animal, preferably a mouse, harboring a transgene encoding amyloid precursor protein (APP) comprising the Arctic mutation (E693G) together with another transgene that enhances Aβx-40 and/or Aβx-42 Arctic peptide and Aβ Arctic protofibril steady-state levels through increased production or impaired clearance or confers enhanced Aβx-40 and/or Aβx-42 Arctic peptide aggregation thereby to confer an early soluble oligomeric and protofibrillar Aβ Arctic peptide-driven pathology, including Aβ aggregation inside neurons of the brain to better reflect the human AD pathology. The

invention discloses both transgenic cells and transgenic mice and progeny thereof made by the method.

The present invention provides a model for Alzheimer's disease and related neurological disorders having pathologies of enhanced Aβx-40 and/or Aβx-42 Arctic peptides and Aβ Arctic protofibril production and an early soluble oligomeric and protofibrillar Aβ Arctic peptide-driven pathology, including such Aβ aggregation also inside neurons of the brain, like in the pyramidal cell layer of CA1 in the hippocampus and in scattered neurons of the lower lamina in the cerebral cortex, to better reflect the human AD pathology.

The present invention provides a method of preparing such a transgenic non-human animal, preferably a mouse, and the transgenic mice made by the method.

The invention further provides the use of such transgenic non-human animals and progeny thereof and/or transgenic cells as a model for a variety of diseases and to be used for drug screening assays and various test compounds, evaluation of diagnostic markers as well as other applications. In one embodiment, a transgene of the APP polypeptide comprises both the Arctic mutation (E693G) and other APP mutations such as KM670/671NL, KM670/671NF, KM670/671NY, KM670/671DL, KM670/671DF, KM670/671DY; KM670/671EL, KM670/671EF or KM670/671EY (APP770 numbering) to enhance both Aβx-40 and Aβx-42 Arctic peptide production in tissues of such transgenic animal. The invention includes the introduction of any of the mentioned APP transgenes (wild-type or containing pathogenic AD mutations) into the endogenous APP alleles.

In multiple further embodiments different other transgenes are introduced in a transgenic nonhuman animal which harbors an APP polypeptide comprising the Arctic mutation (E693G). These transgenes are encoding a heterologous presenilin-1 or presenilin-2 harboring AD pathogenic

mutations to enhance Aβx-40 and/or Aβx-42 Arctic peptide levels through altered γ-secretase cleavage, homologously integrated targeting construct for neprilysin and/or insulin-degrading enzyme (IDE) genes to disrupt these genes through gene ablation (knock-out) to thereby enhance Aβx-40 and/or Aβx-42 Arctic peptide levels by impaired clearance. Finally transgenes harboring the apolipoprotein E, apolipoprotein J (clusterin), α_1-antichymotrypsin (ACT) or fragments thereof to enhance the fibrillization process of Aβx-40 and/or Aβx-42 Arctic peptides are included in the invention. The invention discloses transgenic techniques of introducing such transgenes into a fertilized egg as well as embryonic stem (ES) cell, by techniques known to a person skilled in the art such as microinjection, electroporation, lipofection, or biolistics.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Ethidium-bromide stained DNA gel showing the presence of positive PCR-signal of a DNA-fragment that is of 428bp lengths with upstream (**A**) and of 441bp lengths with downstream (**B**) primer pairs. Genomic DNA from different founder mice have been analyzed and PCR-positive Thy-SwedishArcticAPP founders have been assigned founder line numbers A,B,C and D, as denoted above the gels. DNA molecular weight standard ("mw-std.") shows the lengths of various predefined DNA-fragments. The two primer pairs frames the whole coding region of transgene APP and the basal promoter of the Thy-1 promoter.

Figure 2: Slot–blot phosphor-imager screen reflecting radioactive emission from cRNA-probes hybridized to genomic DNA samples from individual mice from the different founder lines (Thy-SwedishArcticAPP line A, B, C and D) and nontransgenic mice, as denoted for each individual mouse above the corresponding photographic signal (left) and quantitative estimates of these signals to measure copy number for the different founder lines of Thy-SwedishArctic-APPmice (right)

Figure 3: Graph depicting the APP protein with the kunitz domain (hatched) which enables alternative splicing of APP. The Aβ peptides domain (black) resides partly inside the transmembrane domain. The locations of the epitopes of the APP antibodies used in the experiment are indicated. In the APP770 protein isoform the epitopes are located between aa 66-81 (22C11) and aa 672-687 (6E10). The 22C11 antibody detects both human and endogenous murine APP, while the 6E10 antibody detects only human APP. Western blot showing threefold relative overexpression of APP in brain of Thy-SwedishArctic-APP transgenic mouse, founder line B. Coomassie staining ("Cooma.") is a measure of total protein loaded onto the gel (**A**). The presence of human APP and Arctic Aβ peptides in brain of Thy-SwedishArctic-APP transgenic mouse, founder line B ("B") and absence in brain of nontransgenic mouse ("ntr") (**B**).

Figure 4: APP protein in young Thy-SwedishArctic-APP transgenic mouse APP protein expression in the brain of a 1months old Thy-SwedishArcticAPP mouse, line B (**a** and **b**) and a nontransgenic mouse (**c**) stained with 6E10 (epitope 1-16 in Aβ, this antibody is specific for human APP and Aβ). The staining visualizes neuronal distribution of APP protein synthesis in the brain.

Figure 5. Aβ-aggregates are located inside nerve cells in the brain of a Thy-SwedishArctic-APP transgenic mouse. Aβ immunoreactive deposition in PDAPP mouse brain (a commonly used APP mouse) (**A**) and in our Thy-SwedishArcticAPP mouse at 7 months of age (**B**). The arrows points to the pronounced formic acid-resistant Aβ-immunoreactive staining in CA1 pyramidal neurons of Thy-SwedishArctic APP (**B**), which is in sharp contrast to absence of such Aβ-immunoreactive labeling in the PDAPP mouse brain (**A**). Closer inspection reveals that the Aβ-immunostaining is located inside nerve cell resembling inclusions in the pyramidal neurons of CA1 in the hippocampus (**C**) and in scattered nerve cells mainly in the lower lamina of the cerebral cortex (**D**).

Figure 6: Scattergram showing the group mean (line) and distribution among individuals of left hemisphere brain weight as dissected from cohorts of Thy-SwedishArcticAPP and Thy-SwedishAPP transgenic mice at 2 months of age. Thy-SwedishArcticAPP transgenic mice display reduced brain weight (221±9mg; n=9), as compared to Thy-SwedishAPP transgenic mice (239±5mg; n=8), which suggests atrophic changes in the brains of Thy-SwedishArctic APP transgenic mice , as is normally observed in human brain afflicted by Alzheimer's disease pathogenesis.

Figure 7: Extracellular senile plaques in the hippocampus of a Thy-SwedishArcticAPP transgenic mouse at 7 months of age. The Aβ-immunoreactivity was observed with two different antibodies that were specific for the short amino acid fragments in the C-terminal ends of Aβ42 (**a**) and Aβ40 (**b**) and thus do not detect APP or fragments thereof (Näslund et al., 2000). The Aβ-immunoreactivity was resistant to and enhanced by pretreatment with concentrated formic acid. The arrows points to Aβ-immunoreactive deposits which are displayed at higher magnification (**middle images adjacent to a and b**). Combined Congo Red and GFAP-immunostaining shows robust astrogliosis surrounding a compact amyloid plaque (**c**), which display classical gold-green birefringence in polarized light (**d**).

DETAILED DESCRIPTION OF THE INVENTION:

The present invention provides a model for Alzheimer's disease and related neurological disorders having pathologies of enhanced Aβ protofibril formation and intraneuronal Aβ peptide aggregation. The Aβ-immunopositive intraneuronal staining is resistant to pretreatment with concentrated formic acid, which is a typical characteristic of amyloid i.e. Aβ aggregates with a β-sheet structure, and can be localized to the pyramidal cell layer of CA1 in the hippocampus and in scattered neurons of the lower lamina in the cerebral cortex as well as other neurons in the brain. The transgenic mouse model provided by the invention also display reduced brain weight, which suggests

atrophic changes in the brain as is normally observed in human brain afflicted by Alzheimer's disease pathogenesis. The present invention provides a method of preparing such a transgenic non-human animal, preferably a mouse, and the transgenic mice made by the method.

In an embodiment a transgene comprising a polynucleotide sequence a heterologous APP polypeptide comprising the Arctic mutation (E693G) operably linked to a transcription regulatory sequence capable of producing expression of the heterologous APP polypeptide in the transgenic nonhuman animal is used. A promoter can be constitutive or inducible, and can affect the expression of a polynucleotide in a general or tissue-specific manner. Tissue-specific promoters include, without limitation, neuron specific enolase (NSE) promoter, neurofilament light chain (NF-L) and neurofilament heavy chain (NF-H) promoter, prion protein (PrP) promoter, tyrosine hydroxylase promoter, platelet-derived growth factor (PDGF) promoter, thy1-glycoprotein promoter, β-actin promoter, ubiquitin promoter, simian virus 40 (SV40) promoter, and gene-specific promoters such as the APP promoter The amyloid precursor proteins (APP) comprise a group of ubiquitously expressed transmembrane glycoproteins whose heterogeneity arises from both alternative splicing and post-translational processing [Selkoe, D. J. (1994) NCBI accession nr P05067]. Apart from the 751-and 770-residue splice forms which are highly expressed in non-neuronal cells throughout the body, also the 695-residue isoform which is more abundant in neurons. All isoforms are the precursors of various metabolites that result from different proteolytic cleavage induced by physiological or pathological conditions. The APP itself, as used according to the principles of this invention, can be any of the alternative splice forms of this molecule and may be used either as a glycosylated or non-glycosylated form. In a further embodiment the same transgene contains a polynucleotide sequence that harbors APP mutations such as KM670/671NL, KM670/671NF, KM670/671NY, KM670/671DL , KM670/671DF, KM670/671DY, KM670/671EL, KM670/671EF or KM670/671EY (APP770 numbering) to

enhance both Aβx-40 and Aβx-42 Arctic peptide production in tissues of such transgenic non-human animal harboring the Arctic mutation (E693G). In a preferred embodiment the transgene harbors the Arctic mutation (E693G), one of the mutations KM670/671NL, KM670/671NF, KM670/671NY, KM670/671DL, KM670/671DF, KM670/671DY KM670/671EL, KM670/671EF or KM670/671EY (APP770 numbering) and no additional mutations.

In a further embodiment another transgene that enhance Aβx-40 and/or Aβx-42 Arctic peptide production e.g. a transgene encoding a heterologous presenilin-1 or presenilin-2 harboring AD pathogenic mutations in tissues to alter Aβx-40 and/or Aβx-42 Arctic peptide levels by γ-secretase cleavage of such transgenic non-human animal harboring the Arctic mutation (E693G). Such mutations are known in the art and may e.g. be selected from those disclosed on http://www.alzforum.org/res/com/mut/pre/table1.asp (Presenilin-1) and http://www.alzforum.org/res/com/mut/pre/table2.asp (Presenilin-2) as downloaded per 2004-03-09. In a further embodiment a targeting construct homologously integrated into an endogenous chromosomal location so as to enhance Aβx-40 and/or Aβx-42 Arctic peptide levels by impaired clearance e.g. through gene ablation (knock-out) of neprilysin and/or insulin-degrading enzyme (IDE) genes in tissues of such transgenic non-human animal harboring the Arctic mutation (E693G). In a further embodiment another transgene e.g. overexpression of apolipoprotein E, apolipoprotein J (clusterin) or α_1-antichymotrypsin (ACT) to enhance the fibrillization process of Aβx-40 and/or Aβx-42 Arctic peptides and/or Aβ protofibrils in tissues of such transgenic non-human animal harboring the Arctic mutation (E693G). The transgenic non-human animal expressing the Arctic mutation (E693G) and the KM670/671NL mutation under control of said promoter have been found to develop strong intraneuronal Aβ aggregation which is a pathological phenotype that goes beyond previously described APP transgenic mouse models.

The invention further provides non-human transgenic animals, preferably a mouse, which harbors at least one copy of a transgene or targeting construct

of the invention, either homologously or nonhomologously integrated into an endogenous chromosomal location so as to produce Arctic Aβ peptides. Such transgenic animals are usually produced by introducing the transgene or targeting construct into a fertilized egg or embryonic stem (ES) cell, typically by microinjection, electroporation, lipofection, or biolistics. The invention provides nonhuman transgenic animals, preferably a mouse, that have at least one inactivated endogenous APP allele, and preferably are homozygous for inactivated APP alleles, and which are substantially incapable of directing the efficient expression of endogenous (i.e., wild-type) APP. For example, in a preferred embodiment, a transgenic mouse is homozygous for inactivated endogenous APP alleles and is substantially incapable of producing murine APP encoded by a endogenous (i.e., naturally-occurring) APP gene. Such a transgenic mouse, having inactivated endogenous APP genes, is a preferred host recipient for a transgene encoding a heterologous APP polypeptide, preferably a human Arctic mutation and APP polypeptide mutations such as KM670/671NL, KM670/671NF, KM670/671NY, KM670/671DL, KM670/671DF, KM670/671DY, KM670/671EL, KM670/671EF or KM670/671EY (APP770 numbering) to enhance both Aβx-40 and Aβx-42 Arctic peptide production. Such a transgenic mouse, having inactivated endogenous APP genes, is also a preferred host recipient for a transgene encoding a heterologous APP polypeptide, preferably a human Arctic mutation together with another transgene that enhance Aβx-40 and/or Aβx-42 peptide production e.g. a transgene encoding a heterologous presenilin-1 or presenilin-2 harboring AD pathogenic mutations to enhance Aβx-40 and/or Aβx-42 Arctic peptide levels by altered γ-secretase cleavage. Such heterologous transgenes may be integrated by homologous recombination or gene conversion into a nonhuman presenilin-1 or presenilin-2 gene locus, thereby effecting simultaneous knockout of the endogenous presenilin-1 or presenilin-2 gene (or segment thereof) and replacement with the human presenilin-1 or presenilin-2 gene (or segment thereof).

The transgenic animals produce the Arctic Aβ peptides typically in brain tissue. Such animals as well as transgenic cells and progeny thereof are

suitable for use in a variety of disease models and drug screening assays, evaluation of diagnostic markers as well as other applications. As another embodiment of the invention a screening method is provided wherein various test compounds are screened using transgenic cells and/or transgenic non-human animals and/or progeny thereof. Compounds that are found to have an effect on Aβ Arctic peptide expression, or to promote or inhibit any of the diverse biochemical effects of Aβ Arctic peptides and/or aggregated forms of Aβ Arctic peptides such as Aβ protofibrils, are then further tested and used in treatment of Alzheimer's disease and/or related neurological disorders. In accordance with another aspect of the invention, progeny of the invention can be used as starting points for rational drug design to provide ligands, therapeutic drugs or other types of small chemical molecules as well as proteins, antibodies or natural products. Alternatively, small molecules or other compounds as previously described and identified by the above-described screening assays can serve as "lead compounds" in rational drug design.

As such, those skilled in the art will appreciate that the conception upon which this disclosure is based may readily be utilized as a basis for the designing of other structures, methods and systems for carrying out the several purposes of the present invention. It is important, therefore, that the claims be regarded as including such equivalent constructions insofar as they do not depart from the spirit and scope of the present invention.

The invention further relates to cells comprising a transgene encoding amyloid precursor protein (APP) comprising the Arctic mutation and APP mutations that enhance Aβx-40 and Aβx-42 peptide production mutations, such as KM670/671NL, KM670/671NF, KM670/671NY, KM670/671DL , KM670/671DF, KM670/671DY, KM670/671EL, KM670/671EF or KM670/671EY (APP770 numbering). The invention also relates to cells comprising a transgene encoding APP comprising the Arctic mutation together with another transgene that enhance Aβx-40 and/or Aβx-42 peptide production e.g. a transgene encoding a heterologous presenilin-1 or presenilin-2 harboring AD pathogenic mutations. The invention also relates

to cells harboring a targeting construct either homologously or nonhomologously integrated into an endogenous chromosomal location so as to enhance Aβx-40 and/or Aβx-42 Arctic peptide levels by impaired clearance e.g. through gene ablation (knock-out) of neprilysin and/or insulin-degrading enzyme (IDE) genes in cells comprising a transgene encoding APP comprising the Arctic mutation (E693G). The invention also relates to cells comprising another transgene e.g. overexpression of apolipoprotein E, apolipoprotein J (clusterin) or α_1-antichymotrypsin (ACT) to enhance the fibrillization process of Aβx-40 and/or Aβx-42 Arctic peptides and/or Aβ Arctic protofibrils in cells of such transgenic non-human animal harboring the Arctic mutation (E693G).

EXAMPLES

General Methods: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), and in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989). Standard transgenic techniques for introduction of a foreign gene into fertilized eggs from mouse known in the art and not specifically described were generally followed as in Nagy et al., Manipulating the Mouse Embryo: A laboratory manual, Cold Springs Harbor Laboratory, New York (1986, 1994, 2002), ISBN 0-87969-574-9. (**Figures 1 and 2**). General methods in immunohistochemistry: Standard methods known in the art and not specifically described were generally followed as in Stites et al. (eds), Basic and Clinical Immunology (8th Edition), Appleton & Lange, Norwalk, Conn. (1994) and Johnstone & Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, Oxford, 1982 (**Figures 4, 5 and 7**).

Subcloning of expression vectors Thy-SweArcAPP): The transgenic constructs used for this study contain the murine Thy-1 expression vector and human

APP cDNAs. The APP695 isoform, which is predominant APP isoform in the brain, was used. Modifications in human APP cDNA clone (Kang et al., 1987) between NruI(+145nt) and and SmaI(+3100) was made with enzymatic primer extension using the Transfomer mutagenesis kit (Clontech). The following primers were used: CACTCGGTGCC CCGCGCGCGGCCGCCATGCTGCCCGGTTTGGC (SEQ ID NO: 2) and CATAAATAAATTAAATAAAATAACCGCGGCCGCAGAAACATACAAGCTGTCAG (SEQ ID NO: 3) to incorporate flanking NotI-sites and a Kozak sequence for improved initiation of translation.

CAAATATCAAGACGGAGGAGATATCTGAAGTGAATCTGGATGCAGAATTCCGA C (SEQ ID NO: 4) to introduce the KM670/671NL mutation and CAAAAATTGGTGTTCTTTGCAGGAGATGTGGGTTCAAACAAAG (SEQ ID NO: 5) to introduce the E693G mutation. Clones were initially selected through PCR followed by restriction enzyme digestion and the selected clones were checked by DNA sequencing throughout the whole coding region of the amyloid precursor protein (APP). Correct clones were finally digested with NotI, blunt-end ligated into the XhoI-site of the Thy1 expression cassette. The construct DNA was linearized with NotI as to allow the back-bone vector sequences to be removed from the expression cassette. After purification from β-agarose gel (SeaPlaque GTG) with β-agarase (Invitrogen) and phenol-chloroform extraction the linearized DNA construct (2μg/ml) was microinjected into pronuclear oocytes of hybrid mouse line B6-CBA-F1 (B&M, Denmark). The pronuclear microinjection technique is preferred. Transcription units obtained from a recombinant DNA construct of the invention were injected into pronuclei of animal embryos and the obtained founder transgenics were bred to establish the transgenic line.

Genotyping Litters: The resulting offspring were genotyped by cutting tail tips from weanlings, extracting DNA using a Qiagen DNA extraction kit and analyzed with PCR across the coding sequence of APP and the basal promoter of Thy-1 glycoprotein. Two primers pairs were designed Thy-1 Prom (GAATCCAAGTCGGAACTCTT, SEQ ID NO: 6) together with APP-SQ6

(TGTCAGGAACGAGAAGGGCA, SEQ ID NO: 7), and also APP-SQ3 (GCCGACCGAGGACTGA-CCAC, SEQ ID NO: 8) together with APP-SQ7 (GACACCGATGGGTAGTGAA, SEQ ID NO: 9) (**Figures 1**).

Animal care and brain tissue dissection and handling: SwedishArcticAPP transgenic mice (7 month old) were anesthetized with 0.4ml Avertin (25mg/ml) checked for loss of spinal reflexes and then perfused intracardially with 0.9% saline-solution. The brain was prepared and cut in two hemispheres; one of them was immersed in 4% PFA (paraformaldehyde)/1×SPB (Sorensons Phosphate Buffer, 23mM KH_2PO_4, 70.5mM $Na_2HPO_4 \times 2H_2O$, 5mM NaN_3, pH7.4) over night, 4°C. Thereafter the brain was sequentially transferred and immersed in 10%, 20% and 30% (weight/volume) Sucrose/0.1×SPB-solution each over night. The sucrose procedure was done to better preserve tissue morphology following freezing. The brain was kept in 30% sucrose-solution until the cryostat sections were cut (**Figure 4, 5 and 7**).

Protein analysis: The left hemispheres of the brains were dissected from the different founder lines and weighed (**Figure 6**) (as well as the other organs measured). The brain tissue was extracted in 0.2% Tween-20 in 1xPBS with protease inhibitor tablets (cat 1836153, Roche, one tablet is tablet is sufficient for 10ml extraction solution). The extraction ratio was 1:10 (tissue weight: extraction buffer) and the tissue was extracted by 2x10 strokes on ice. The extraction solutions were centrifuged at 17900g at 10°C for 15min. The supernatants were divided into aliquots and stored at -20°C. All samples (~40µg protein each) were denatured by adding 1% mercaptoethanol and 1×Sample buffer (final concentration), the samples were mixed and boiled for 5min and then loaded on 4-20% Tris-Glycine gel (InVitrogen). 1×Sample buffer contains 10% Glycerol, 2% SDS, 50mM Tris-HCl and Bromophenol blue (diluted x40 from a 1.5% stock). The SDS-PAGE running buffer used includes 250mM Tris-base, 1.9M Glycine and 35mM SDS (Sodium Dodecyl Sulfate). The gel was run at 95V. A Nitrocellulose filter was prewet in ddH_2O

and then equilibrated in 1×Transfer-buffer (30mM Tris-base, 230mM Glycine, pH8.3) with 20% methanol. The transfer set was assembled in transfer-buffer and the transfer was run at 55V, 4°C over night. Prior to the antibody incubations the nitrocellulose-filter was boiled in 1xPBS for 5min, to stabilize and increase the exposure of epitopes in Aβ. The filter was then blocked in freshly prepared 1% w/v nonfat dry milk, 0.1% Tween-20 in 1×TBS-buffer (100mM Tris base, 0.9% NaCl, pH 7.5) for 1hr at room-temperature. After blocking, the filter was incubated with primary antibody (0.5µg/ml 6E10 or 2µg/ml 22C11) in 0.1% Tween-20 in 1×TBS-buffer for 1hr at room-temperature. This was followed by washing 3-4 times (5min) in room-tempered 0.1% Tween-20 in 1×TBS-buffer. The secondary antibody, 0.2µg/ml anti-mouseIgG/IgM-HRP (Pierce), in room-tempered 1% w/v nonfat dry milk, 0.1% Tween-20 in 1×TBS-buffer and the filter was incubated in this solution for 30min. The filter was then washed three more times in 0.1% Tween-20 in 1×TBS-buffer, and last there was a final rinse in 1×TBS-buffer without Tween before the 5min incubation in SuperSignal (Pierce-ECL). All incubations were let to proceed on a shaking platform. The blot filter was finally incubated against an ECL-Hyperfilm (Amersham) (**Figures 3**).

Immunohistochemistry: The brain hemispheres from the founder lines mounted on a chuck were cut to 14µm sections on a cryostat. For the immunostaining a M.O.M. kit from Vector was utilized. The frozen fixed tissue sections were incubated in prewarmed citrate-buffer (25mM, pH7.3) for 5min at 85°C. This was followed by a rinse in 1×PBS. After that the sections were incubated with H_2O_2 (0.3%) in 50% DAKO-block/50% 1×PBS for 15min at room-temperature to block endogenous peroxidase activity. The brain sections were once again rinsed in 1×PBS before the incubation with M.O.M. Mouse IgG Blocking Reagent for 1hr to block unspecific binding. Then the sections were permeabilized with 1×PBS (pH7.4) +0.4% Triton X-100) for 5min and briefly rinsed twice in 1×PBS (pH7.4) to increase the surface tension. M.O.M. Mouse Diluent was used for the 5min incubation to block unspecific binding and excess were wiped away. Incubation with

0.2µg/ml 6E10, 14µg/ml GFAP (clone G-A-5; 1x1500) 1.5µg/ml Aβ42 and 1.7µg/ml Aβ40 antibodies (primary antibodies) in MOM-diluent/ 0.1% Triton X-100 was let to proceed for 1hr. After another wash in 1xPBS buffer the sections were incubated with M.O.M. Biotinylated Anti-mouse or Anti-rabbit IgG reagent in M.O.M. Diluent/0.1% Triton X-100 for 8min. The sections were once more rinsed in 1×PBS buffer. A 30min long incubation with the M.O.M. kit ABC-complex (avidin-biotin-complex) were let to proceed, this was followed by a rinse in 1xPBS. Thereafter a horse radish peroxidase based substrate kit (NOVA Red, Vector) was used to develop the staining 10min. Finally the sections were briefly washed in ddH_2O, dehydrated in 70%, 95%, 99.5% EtOH, allowed to air-dry, dehydrated in Xylene and mounted in DPX (Dibutyl Phthalate Xylene, VWR) mounting medium for light microscopy. All the incubations above were carried out in room-temperature and on a shaking platform (**Figure 4, 5 and 7**). Congo Red staining was accomplished by incubating tissue sections with saturated alkaline sodium chloride solution (10mM NaOH) for 20min followed by Congo Red (0.2% w/v) in saturated alkaline sodium chloride solution (10mM NaOH) for 15min and dehydration in 70%, 95%, 99.5% EtOH. Tissue sections were allowed to air-dry, dehydrated in Xylene and mounted in DPX (Dibutyl Phthalate Xylene, VWR) mounting medium for light microscopy under polarized light (**Figure 7**).

RESULTS

PCR screening: The results from PCR genotyping are seen to the right (Fig.1). Both sets of primers identified 4 founder mice (out of 13) having the mThy1-SwedishArctic-hAPP construct and these four founder lines were established; Thy-SwedishArcticAPP lines A-D. DNA-fragments of 428bp lengths with upstream (**A**) and of 441bp length with downstream (**B**) primer pairs could be detected. Offspring from each founder line were genotyped the same way (**Figure 1**).

Slot blot: Copy numbers were analyzed on individual transgene positive offspring using slot blot. The four Thy-SwedishArcticAPP founder line incorporated varying number of DNA copies, with founder line B having the highest copy number (41±2), taking into account that the nontransgenic mice have two copies of the endogenous Thy1 gene **(Figure 2).**

Western blot: Human APP and Aβ synthesis from brain extracts of the different Thy-SwedishArctic founder lines are shown. The drawing illustrates the amyloid precursor protein (APP) and the epitopes within APP that are targeted by the antibodies. In the APP770 protein isoform, the targeted epitopes are amino acids 66-81, for 22C11, and amino acids 672-687, for 6E10. The intensity of the spots has been analyzed with the Scion Image software and relative APP overexpression in the different founder lines has been calculated. Equal loading of the gels has been confirmed with Coomassie straining and total protein analysis. The relative APP expression can be estimated with antibody 22C11 which enables detection of both endogenous murine APP and human transgene APP. In contrast antibody 6E10 only detects human transgene APP and Aβ peptides. Thy-SwedishArcticAPP founder line B was found to display 3-fold APP-overexpression (**Figure 3**).

Immunohistochemistry: The results from the APP immunohistochemistry are presented is seen in a one month old Thy-SwedishArcticAPP, founder line B mouse (**Figure 4a-b**), while only diffuse background staining is apparent in a nontransgenic littermate (**Figure 4c**). We analyzed neuropathology in the mice of 7month old mice, Thy-SwedishArcticAPP, founder line B. We find intraneuronal Aβ-immunopositive inclusions in the pyramidal cell layer of CA1 in the hippocampus and in scattered neurons of the lower lamina in the cerebral cortex in Thy-SweArcAPP transgenic mice (line B) at 7 months of age (**Figure 5B-D**). The Aβ-immunopositive staining is resistant to pre-treatment with concentrated formic acid, which is a typical characteristic of amyloid i.e. Aβ aggregates with a β-sheet structure. Such pathology has never been previously reported in single APP transgenic mice e.g. the PDAPP-model (**Figure 5A**), and not even in crossed PS/APP transgenic mice (e.g. Holcomb et al., 1998). Extracellular senile plaques were also present in the caudal part of hippocampus of Thy-SweArcticAPP transgenic mouse at this age, as shown with Aβ42 and Aβ40 specific antibodies (**Figure 7a-b**). The Aβ-immunoreactivity was resistant to and enhanced by pretreatment with concentrated formic acid. The arrows (**in Figure 7a-b**) points to Aβ-immunoreactive deposits which are displayed at higher magnification (**middle images adjacent to a and b**). Combined Congo Red and GFAP-immunostaining shows robust astrogliotic reaction surrounding a compact amyloid plaque (**Figure 7c**), which display classical gold-green birefringence in polarized light (**Figure 7d**).

Brain weight: The brains were dissected and divided into its two hemispheres. Scattergram showing mean and distribution among individuals of left hemisphere brain weight. The brain tissue was later biochemically analysed for human APP and Aβ synthesis. The left hemisphere was initially weighed on a balance, to serve as a measure of atrophic degeneration of the brain **(Figure 6)**.

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CLAIMS

1. A transgenic cell or non-human animal expressing at least one transgene comprising a DNA sequence encoding a heterologous Amyloid Precursor Protein (APP) comprising the Arctic mutation (E693G) and at least one other mutation which increases levels of soluble Aβx-40 and/or Aβx-42 peptides and/or enhances formation of soluble oligomeric Aβ forms including Aβ protofibrils.

2. The cell or animal according to claim 1, wherein the transgene is integrated in the genomic DNA.

3. The cell or animal according to claim 1 or 2, wherein said transgene is operably linked to a promoter effective for expression of said gene in the brain tissue of said animal.

4. The cell or animal according to any of claims 1-3 wherein the endogenous APP is non-expressive.

5. The cell or animal according to any of claims 1-4, wherein the at least one other mutation is one of the APP mutations KM670/671DF, KM670/671DY, KM670/671EF or KM670/671EY.

6. The cell or animal according to any of claims 1-4, wherein the at least one other mutation is one of the APP mutations KM670/671NL, KM670/671NF, KM670/671NY, KM670/671DL or KM670/671EL

7. The cell or animal according to any of claims 1-6, wherein said transgene is combined with a human presenilin-1 and/or presenilin-2 transgene harboring at least one mutation causing Alzheimer's disease.

8. The cell or animal according to any of claims 1-7, additionally comprising a homologously integrated targeting construct for at least one

of the neprilysin or insulin-degrading enzyme (IDE) genes, disrupting this gene through gene ablation (knock-out).

9. The cell or animal according to any of claims 1-8, additionally comprising at least one transgene comprising a DNA sequence encoding apolipoprotein E, apolipoprotein J (clusterin), α_1-antichymotrypsin (ACT) or fragments thereof.

10. The animal according to any of claims 1-9 wherein the transgenic animal is a rodent, preferably a mouse.

11. The cell according to any of claims 1-10 that is a stem cell, preferably a rodent stem cell, most preferably a mouse stem cell.

12. The use of a cell or an animal according to any of claims 1-11 for screening for agents useful for treating, preventing or inhibiting Alzheimer's disease.

13. The use of a cell or an animal according to any of claims 1-11 for screening for diagnostic agents for Alzheimer's disease.

ABSTRACT

The present invention relates to a transgenic cell or non-human animal expressing at least one transgene comprising a DNA sequence encoding a heterologous Amyloid Precursor Protein (APP) comprising the Arctic mutation (E693G) and at least one other mutation which increases levels of soluble Aβx-40 and/or Aβx-42 peptides and/or enhances formation of soluble oligomeric Aβ forms including Aβ protofibrils. It also relates to use of the cell or animal in screening for therapeutic or diagnostic agents useful in treatment or diagnosis of Alzheimer's disease.

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Nilsson, Lars
Lannfelt, Lars
Gellerfors, Pär

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Figure 1

Thy-SwedishArcticAPP founder lines

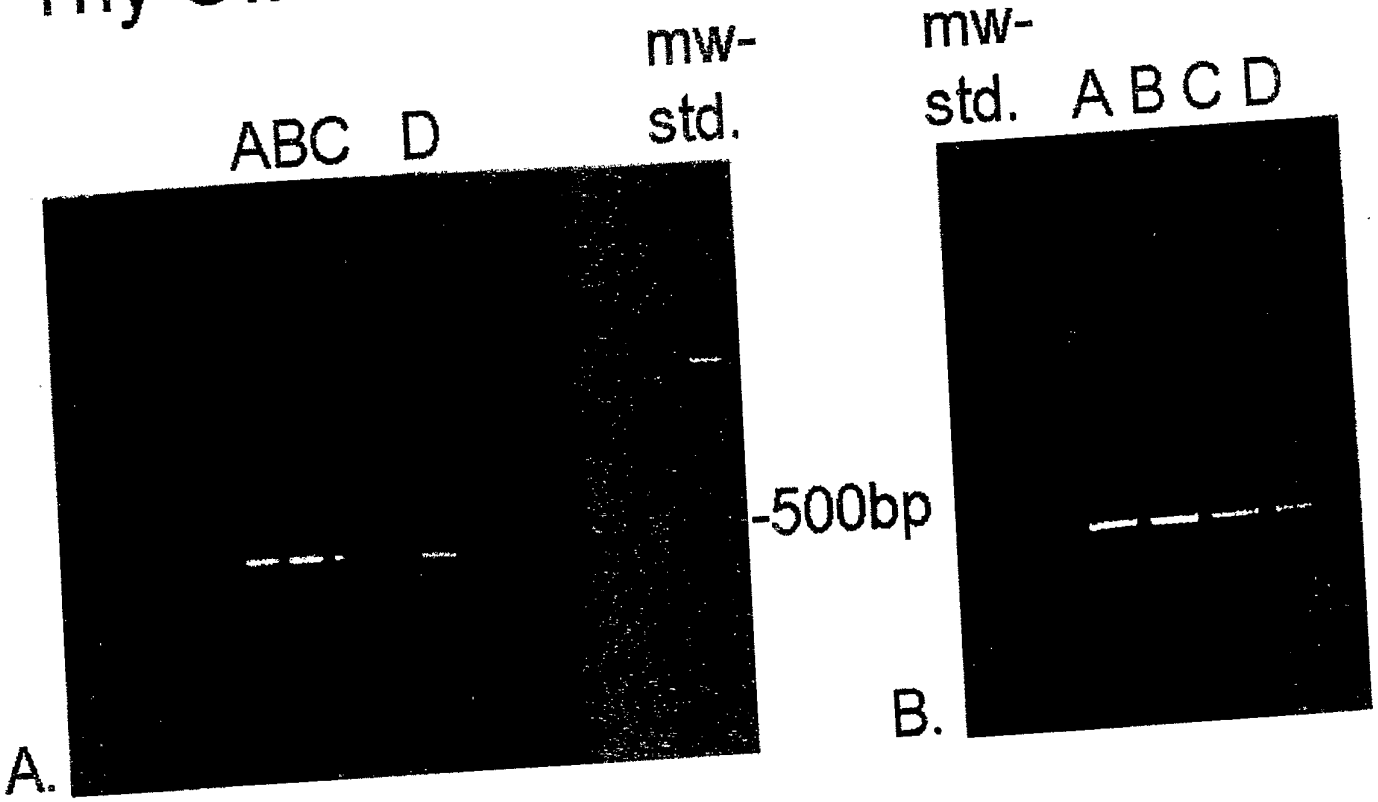
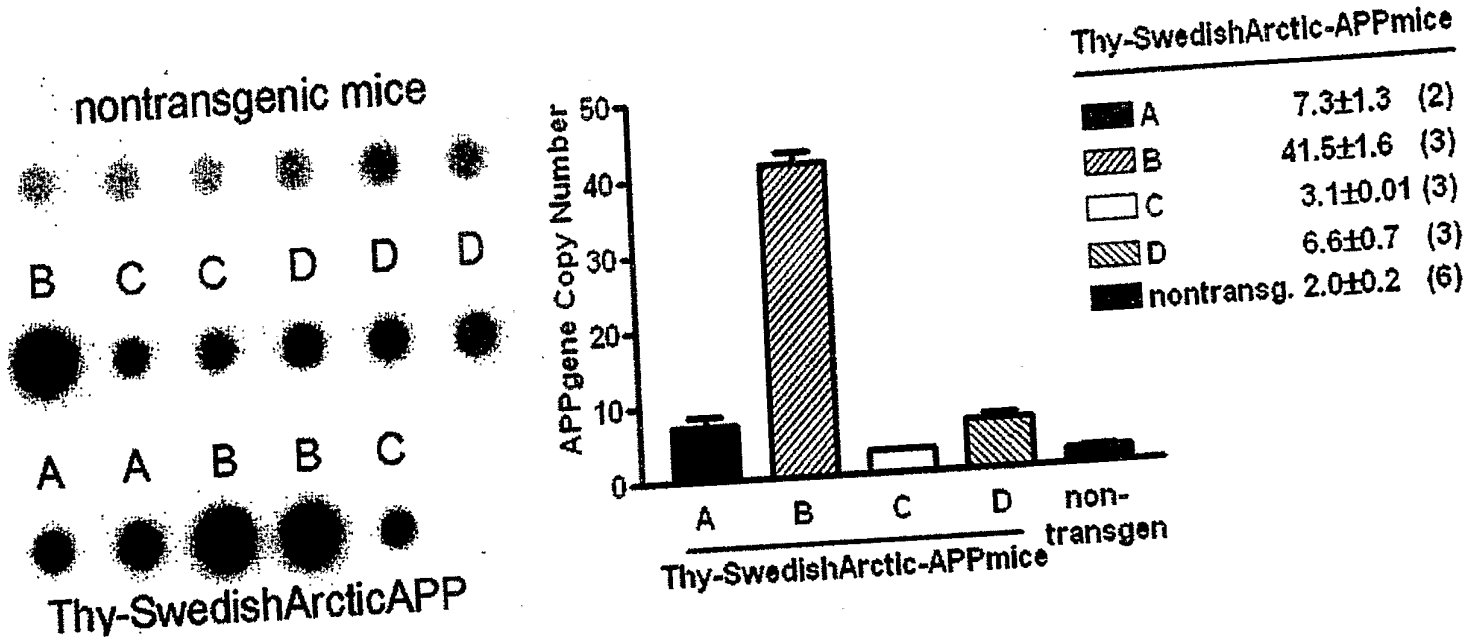


Figure 2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30
20
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Thy-SwedishArctic-APPmice
Thy-SwedishArctic-APPmice
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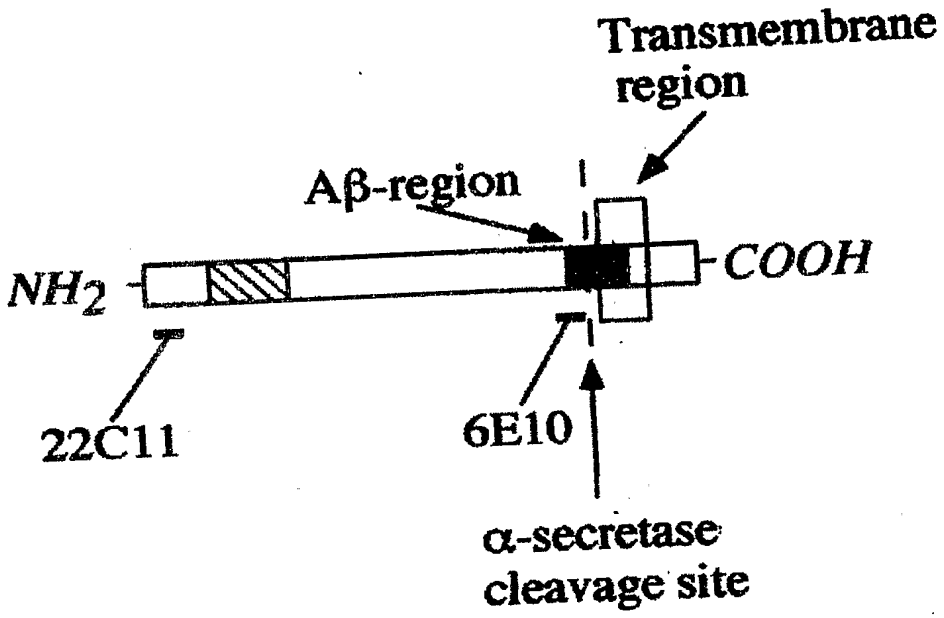
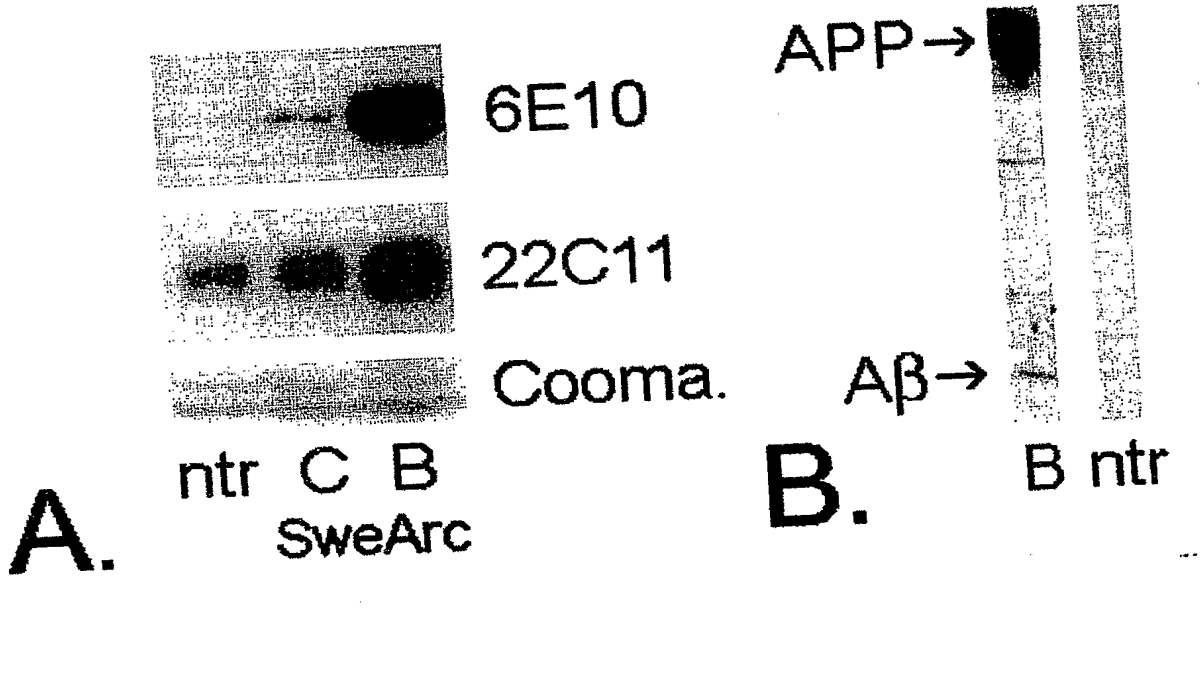
Figure 3





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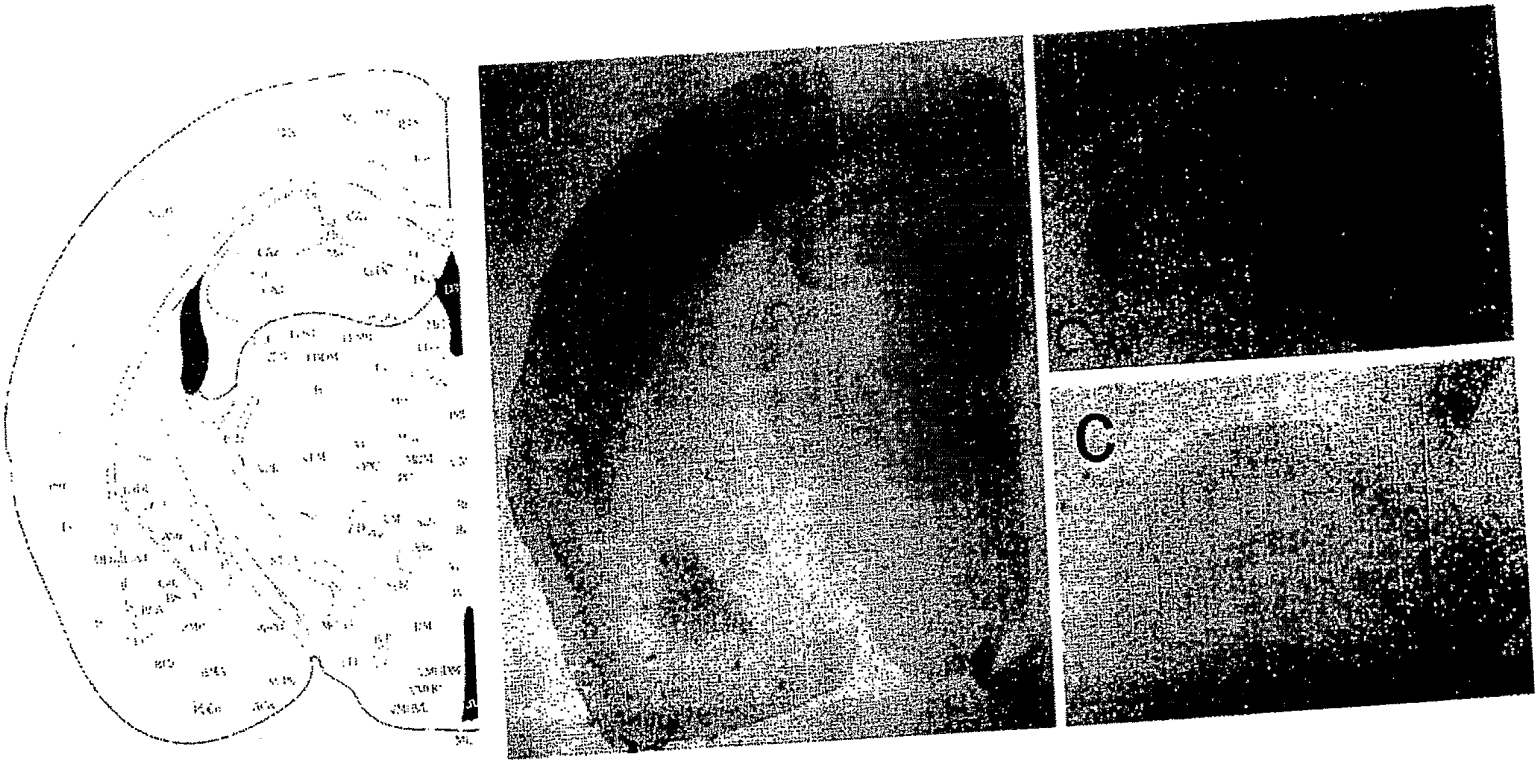

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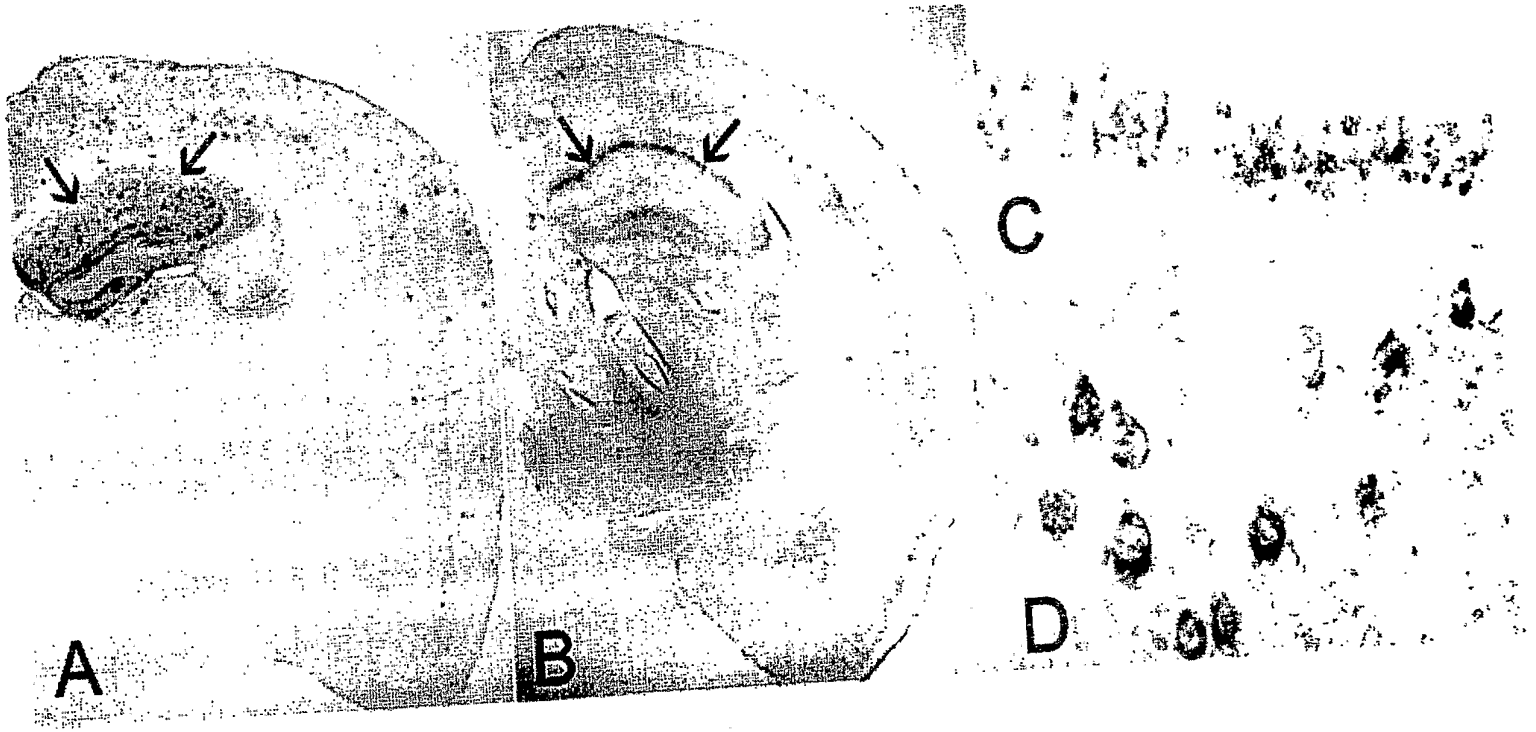

Figure 6

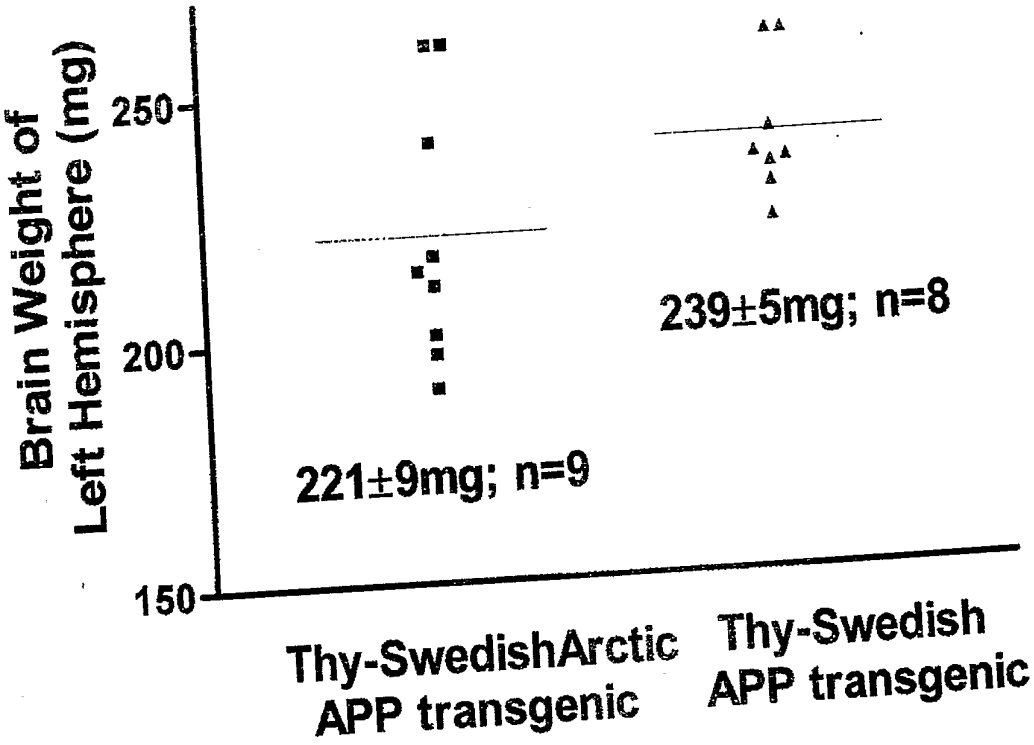
Brain Weight of Left Hemisphere (mg)
250
200
150
221±9mg; n=9
239±5mg; n=8
Thy-SwedishArctic APP transgenic
Thy-Swedish APP transgenic

Figure 7